Mechanical Strain Influences Preosteoclasts Apoptosis through Mitochondrial Pathway

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Abstract

Background: Osteoclasts, the key differentiated cells mainly responsible for bone resorption, are sensitive to mechanical strain. However, the effect of mechanical strain on osteoclasts apoptosis is poorly understood.

Objectives: To investigate the effects of mechanical strain on pre-osteoclast apoptosis in vitro.

Methods: After treatment with osteoclast-inductive medium, the pre-osteoclastic RAW264.7 cells were subjected to mechanical strains of physiological 2500 micro strain (με) and pathologic 5000 με respectively. Then the apoptosis was detected with Annexin V-FITC/PI binding assay and colorimetric assay of caspase-3 activity, mitochondrial membrane potential ($\Delta\psi_m$) was assayed with flow cytometry, and apoptotic proteins were detected with western blots.

Results: The mechanical strain of 2500 με decreased pre-osteoclast apoptosis ratio, caspase-3 activity, elevated $\Delta\psi_m$ and the level of Bcl-2, down-regulated the level of caspase-3 and cytochrome C in cytoplasm. The pre-osteoclasts subjected to 5000 με strain showed no evident difference compared to unstrained group. Additionally, pretreatment with cyclosporine A, an inhibitor of mitochondrial permeability transition pore (mPTP), elevated $\Delta\psi_m$ and lowered mechanical strain of 2500 με reduced apoptosis ratio.

Conclusion: Physiological mechanical strain of 2500 με inhibited pre-osteoclast apoptosis, and the 5000 με strain had no significant effects on apoptosis. The physiological strain influenced pre-osteoclast apoptosis through mPTP/ cytochrome C of the mitochondrial pathway.

Keywords

Mechanical Strain, Osteoclast, Apoptosis, Mitochondria, Cytochrome C

Introduction

Physiological mechanical strain leads to bone homeostasis when bone resorption is equal to bone formation; overload promotes bone gain; and disuse results in bone loss [Adams (2003)]. Bone modeling and remodeling are regulated by osteoclastic bone resorption and osteoblastic bone formation [Akiyama et al. (2008), Boyle et al. (2003)]. The balance between the resorption and the formation is of critical importance to many bone diseases such as osteoporosis and osteopetrosis [Cossarizza et al. (1993), Frost (2003)].

Osteoclast is a hematopoietic and branches from the monocyte-macrophage lineage during the differentiation process. Although it has been reported that many hormones and cytokines promote osteoclast formation such as 1, 25-dihydroxyvitamin D3, transforming growth factor –α, interleukin, RANKL and M-CSF are the crucial formation factors in the process of osteoclast formation [Frost (2001), Green & Reed (1998)]. Some studies have demonstrated that mechanical loading influence bone marrow-derived pre-osteoclast-like cells activity, osteoclastogenesis and bone-resorbing activity of osteoclasts or pre-osteoclast cells [Gross et al. (1999), Hao et al. (2013), Jee (2000), Julian & Matthew (2005)]. Recently, our study indicated that osteoclastogenesis and osteoclast-related gene expression of RAW264.7 cells stimulated with mechanical strains at different magnitudes, are different [Kowaltowski et al. (2001)].

Osteoclasts have a finite life span, and undergo spontaneous apoptosis [Kurata et al. (2001)]. Tiny changes in osteoclast apoptosis can cause large changes in bone remodeling [Liu & Li (2010), Li et al. (1997), Madesh & Hajnoczky (2001)]. As osteoclast is mechano-responsive, mechanical strain should have affect on osteoclasts apoptosis. However, the influence of mechanical strain on osteoclasts apoptosis is still poorly understood.

Since mechanical strain has a great influence on osteoclast, it was hypothesized that mechanical strain is involved in the regulation of osteoclast apoptosis. In

this study, the pre-osteoclastic RAW264.7 cells were stimulated with different mechanical strains, and the effect of mechanical strains on apoptosis was investigated. In addition, the involvement of mitochondrial pathway in apoptosis of RAW264.7 cells subjected to mechanical strain was also studied.

Materials and Methods

Materials

Mouse RAW264.7 cells were obtained from cell culture center of Peking Union Medical College (Beijing, China). Alpha minimal essential medium (α-MEM) was purchased from Invitrogen (Invitrogen, USA). Receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) were purchased from Peprotech (Rocky Hill, NJ, USA). Annexin V/PI detection apoptotic Kit and ECL detection Kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). JC-1 (5,5′,6,6′ -tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) Mitochondrial membrane potential test Kit and caspase-3 Colorimetric assay Kit were purchased from Nanjing Biobox Biotechnology Co., Ltd (Nanjing, China). Mitochondria/cytoplasm Isolation Kit from Applygen Technologies Inc (Beijing China), Caspase-3, cytochrome C, Bcl-2 and GAPDH antibodies were provided by Wuhan Boster Bioengineering Co., Ltd. (Wuhan, China).

RAW264.7 Cell Culture

Pre-osteoclastic RAW264.7 cells, a mouse monocytic cell line which can be induced into osteoclasts, were maintained in α-MEM, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The osteoclastic differentiation from RAW264.7 cells was induced with osteoclast-inductive medium (α-MEM containing 50 ng/mL M-CSF and 50 ng/mL RANKL). Mature osteoclasts were observed after 3 days of inducement.

Application of Mechanical Strain to Cells

The application of mechanical strain on the cells was conducted with a specially designed four-point bending device described and used previously [Martin (2000), McAllister et al. (2000)]. RAW264.7 cells were seeded at the density of 10^4/ cm^2 in the cell culture dishes (cultured in osteoclast -inductive medium for 3 days), and then randomly divided into 3 groups. One group of cells was subjected to mechanical strain of physiological 2500 μs at 0.5 HZ for 1 hour per day; another group of cells was subjected to mechanical strain of 5000 μs at 0.5 HZ for 1 hour per day; and the third group of cells was not subjected to mechanical strain as a control group. The mechanical loadings continued for 3 days.

Analysis of Cell Apoptosis

The extent of apoptosis was measured through Annexin V-FITC apoptosis detection kit as described by the manufacture's instruction. After mechanical strain, osteoclasts were collected, washed twice with PBS, gently re-suspended in Annexin V binding buffer and incubated with Annexin V-FITC/PI in dark for 15 min and analyzed by flow cytometry. The fraction of cell population in different quadrants was analyzed using quadrant statistics.

Assay of caspase-3 activity was performed according to the protocol of manufacturer of a specific kit. Each protein sample was conducted in triplicate with parallel 3-well culture plates to ensure accurate results. Using the professional software Curve Exert 1.3 provided by Cusabio Biotech Co., LTD, USA, a standard curve was made for calculation of caspase-3 activity. The results were presented as the percentage of activity change, compared to the control.

Measurement of Mitochondrial Membrane Potential ($\Delta\psi_M$)

$\Delta\psi_m$ was assessed in preosteoclasts using a fluorescent probe,5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) according to the protocol of manufacturer, and the assay was described previously [Nakahama (2010)]. After mechanical strain, the pre-osteoclasts were incubated with JC-1 working buffer at 37°C and 5% CO_2 for 15 min, and then collected, washed with the staining binding buffer and analyzed by flow cytometry. In this assay, depending on the membrane potential, JC-1 is capable of forming J-aggregates. The higher the membrane potential was, the more the J-aggregates was, and the less the JC- 1 was. When excited, J-aggregates and JC-1produced red and green fluorescence, respectively. So the relative $\Delta\psi_m$ was showed as ratio of red fluorescence intensity to green fluorescence intensity (red/green).

Western Blot Analysis

After mechanical strain, the cells were washed with cold PBS. Following treatment with Mitochondria Isolation Kit according to manufacturer's protocol, the total protein was prepared, motochondria protein and cytopl asm protein were isolated. Fifty micrograms of protein

sample were separated by 15% SDS- polyacr ylamide gel electrophoresis and transferred into a PVDF membrane. The membrane was incubated with primary antibodies in 5% nonfat milk in PBS with 0.1% Tween-20 respectively, followed by incubation with horseradish peroxidase-conjugated IgG as the secondary antibody. The chemiluminescence reaction was carried out using an ECL kit.

Statistical Analysis

Data expressed as mean ± SD were normalized to control. Statistical analysis was performed with ANOVA using SPSS13.0 software. P values less tha n0.05 were considered statistically significant.

Results

Mechanical Strain of 2500 MS Inhibited Preosteoclast Apoptosis

The result of Annexin-V/PI binding assay indicated that the mechanical strain of 2500 μs reduced the apoptosis ratio of the preosteoclasts. Compared with control group (unstrained group), the 5000 μs strain nearly has no impact upon apoptosis ratio (Figure 1A). In addition, 2500 μs strain reduced caspase-3 activity and 5000 μs had no effect on the activity, which confirmed that the 2500 μs inhibited preosteoclast apoptosis (Figure 1B).

Mechanical Strain Affected on Expression of Apoptotic Proteins

Bcl-2, cytochrome C and caspase-3 are representative apoptotic proteins. In this study, the protein levels of cytochrome C in cytoplasm protein, Bcl-2 and caspase-3 in total protein, were assayed with western blot. As shown in Figure 2, 2500 μs strain led to a significant decrease in the protein level of cytochrome C and caspase-3, and a significant increase in the protein level of Bcl-2. But 5000 μs strain had no significant effect on these three proteins.

Mechanical Strain of 2500 MS Reducing Apoptosis Ratio Was Mediated By Mitochondrial Permeability Transition Pore (mPTP)

The opening of the mPTP, resulted in loss of $\Delta\psi_m$, swelling of the mitochondrial matrix, and consequent rupture of the outer mitochondrial membrane and the release of apoptotic proteins [Ozaki et al. (1997), Rodan & Martin (2000)]. In this study, the 2500 μs strain (not 5000 μs) elevated $\Delta\psi_m$ and reduced apoptosis ratio (Figure 1A, C), which suggested that mPTP involved in mediating mechanical strain- reduced apoptosis.

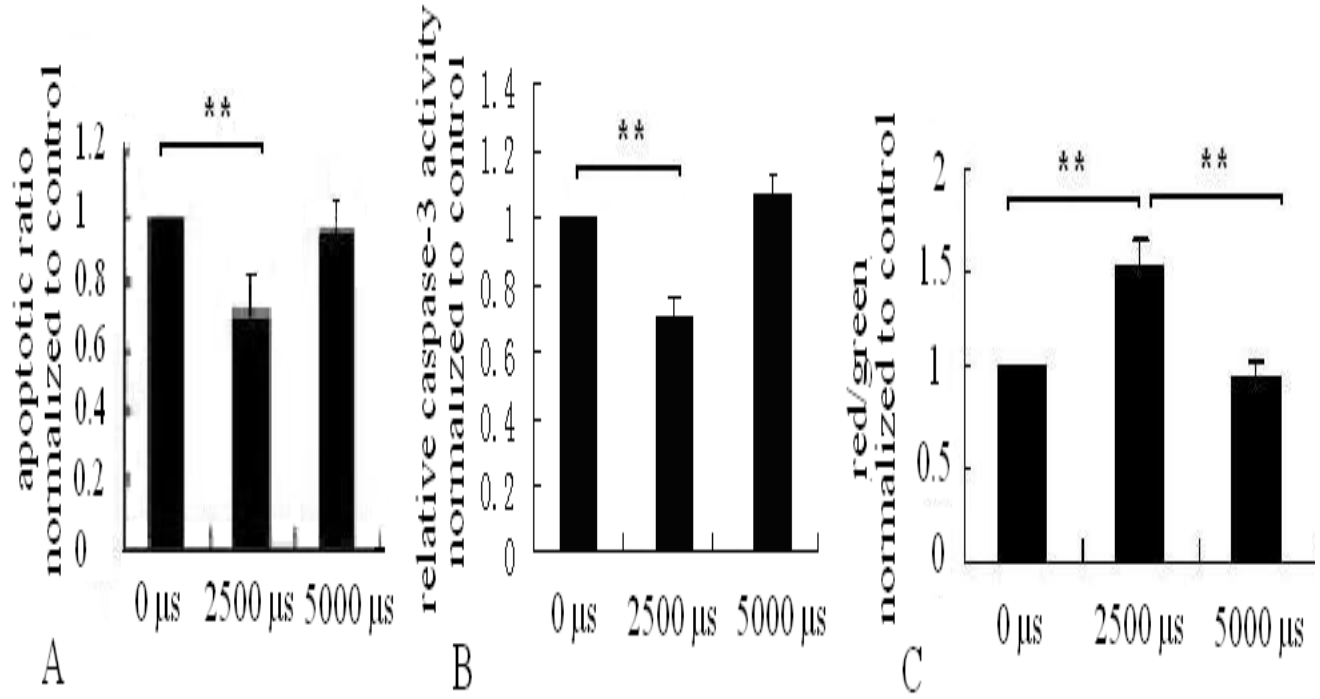

FIGURE 1 A ANALYSIS OF RAW 264.7 CELL APOPTOSIS AND ASSAY OF MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_M$).

The mechanical strain of 2500 μs reduced apoptotic ratio and caspase-3 activity, and The 5000 μs strain had no effect on cell apoptosis (A, B), 2500 μs strain elevated $\Delta\psi_m$ and 5000 μs strain had no effect (C) (*p <0.05, **p<0.01, between indicated groups)

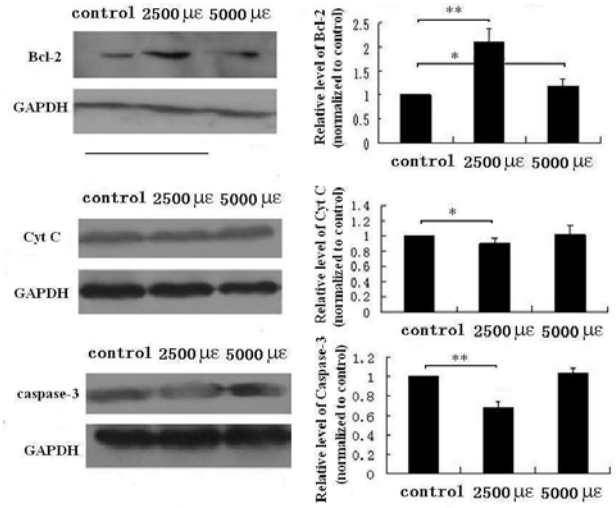

FIGURE 2 WESTERN BLOT ANALYSIS OF BCL-2, CYTOCHROME C AND CASPASE-3.

The mechanical strain of 2500 μs decreased the level of cytochrome C in cytoplasm protein, caspase-3 level in cells total protein, and increased Bcl-2 (anti-apoptosis protein). The 5000 μs strain did not affect on these proteins evidently. (*p <0.05, **p<0.01, between indicated groups)

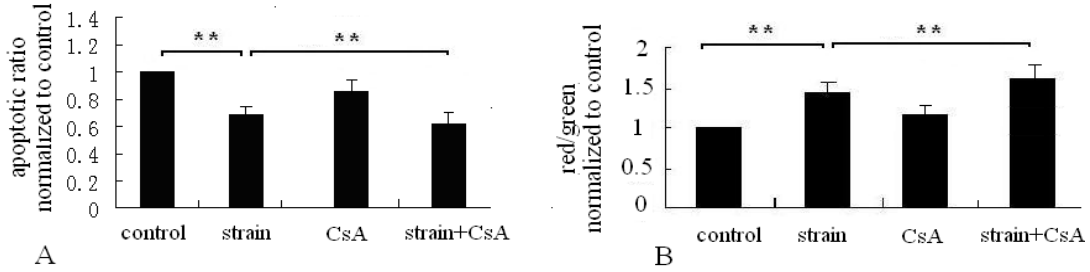

FIGURE 3 APOPTOTIC RATIO AND $\Delta\Psi_M$ OF CELLS PRETREATED WITH CYCLOSPORIN A (CSA), A MITOCHONDRIAL PERMEABILITY TRANSITION PORE (MPTP) INHIBITOR.

Cells were pretreated with 10 μM CsA for 30 min before strain then strained in the presence or absence of 10 μM CsA. Pretreatment of CsA elevated $\Delta\psi_m$, lowered apoptotic ratio, and caused apoptotic ratio to become lower than strained group. (*p <0.05, **p<0.01, between indicated groups)

After pretreatment with cyclosporine A, an inhibitor of mitochondrial permeability transition pore (mPTP), the $\Delta\psi_m$ was elevated, and apoptosis ratio was lowered (Figure 3). The effect of the 2500 μs mechanical strain on apoptosis (lowering ratio and elevating $\Delta\psi_m$) was enhanced. The results confirmed that mechanical strain of 2500 μs reducing apoptosis ratio was mediated by mPTP.

Discussion

The 100-2500 μs span includes the adapted and mild overload windows, and these physiological strains increase bone mass and strength. The 3000-25000 μs span is called pathologic overload window, where microdamage appears and the formation of woven bone is evoked [Rubin et al. (1999), Rubin et al. (2002c), Saelens et al. (2004)]. According to the theory and our previous study [McAllister et al. (2000)], the magnitude of 2500 μs was selected as representative of physiological strain, 5000 μs as representative of pathologic strain.

Although previous studies indicated that mechanical strain plays a great role in osteoclast/preosteoclast differentiation, growth and function [Gross et al. (1999), Hao et al. (2013), Jee (2000), Julian & Matthew (2005), Kowaltowski et al. (2001)], the effect of mechanical strain on osteoclasts apoptosis is still poorly understood. Our previous study showed the influences of different mechanical strains (2500 μs and 5000 μs) on osteoclast apoptosis [Suzuki et al. (2008), Takeuchi et al. (2008)], but the mechanism and signal pathway involved in strain-mediated apoptosis is unexplored yet.

Mitochondrial pathway is one of important pathways controlling apoptosis [Tang et al. (2004), Wang et al. (2001)]. The activation of the pathway is associated with low expression of Bcl-2 which is an anti-apoptosis protein, collapse of mitochondrial membrane potential, release of cytochrome C from mitochondria to the cytoplasm and a high expression of caspase family such as caapase-3 [Ozaki et al. (1997), Rodan & Martin

(2000), Weinstein & Manolagas (2000)].

In this study, it has been found that 2500 μs physiological mechanical strain reduced preosteoclast apoptosis ratio, caspase-3 activity, up-regulated the level of Bcl-2, down-regulated the level of caspase-3 and cytochrome C in cytoplasm. The reduction of Bcl-2, the release of cytochrome C in mitochondria to cytosol results in aopptosis, and caspase-3 is an important enzyme initiating aopptosis [Ozaki et al. (1997), Rodan & Martin (2000)]. The result suggested that mitochondrial pathway was involved in strain-mediated preosteoclast apoptosis.

In this study, pretreatment with cyclosporine A (an inhibitor of mPTP) elevated $\Delta\psi_m$ and enhanced the effect of the 2500 μs mechanical strain on apoptosis (apoptotic ratio become lower). Cytochrome C release is associated with the opening of the mPTP [Xu et al. (2012), Yan et al. (2012)]. The elevation of $\Delta\psi_m$ could be an indicator of the closing of mPTP [Zelzer & Olsen (2003)]. In our study, physiological mechanical strain elevated $\Delta\psi_m$, which indicated that the strain promoted the closing of mPTP. Therefore, these results confirmed that the mechanical strain influences preosteoclasts apoptosis through mitochondrial pathway.

Conclusion

Physiological mechanical strain of 2500 μs inhibited preosteoclast apoptosis, and the 5000 μs strain had no significant effects on apoptosis. The physiological strain influenced preosteoclast apoptosis through mPTP/cytochrome C of the mitochondrial pathway.

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